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(71) Applicant (*for all designated States except US*): THE GOVERNMENT OF THE UNITED STATES OF AMERICA, as represented by THE SECRETARY OF HEALTH AND HUMAN SERVICES, NATIONAL INSTITUTES OF HEALTH [US/US]; Office of Technology Transfer, Suite 325, 6011 Executive Boulevard, Rockville, MD 20852-3804 (US).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): KLEINMAN, Hynda, K. [US/US]; National Institute of Dental and Craniofacial Research NIH, Building 30, Room 433, 30 Convent Drive, MSC-4370, Bethesda, MD 20892-4370 (US). GOLDSTEIN, Allan [US/US]; George Washington University, Dept. of Biochemistry, 2300 I Street, N.W., Washingotn, DC 20037 (US). MALINDA, Katherine, M. [US/US]; National Institute of Dental and Craniofacial Research NIH, Building 30, Room 433, 30 Convent Drive, MSC-4370, Bethesda, MD 20892-4370 (US). SOSNE, Gabriel [US/US]; 25341 Ronald Court, Oak Park, MI 48237 (US).

(74) Agent: WETHERELL, John, R. Jr.; Fish & Richardson P.C., Suite 1400, 4225 Executive Square, La Jolla, CA 92037 (US).

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(54) Title: THYMOSIN $\beta 4$ PROMOTES WOUND REPAIR

(57) Abstract

The present invention relates to methods for promoting tissue repair, angiogenesis and cell migration. The method of the invention utilizes thymosin $\beta 4$ ($T\beta 4$) peptide to promote tissue repair, angiogenesis and cell migration. The invention further relates to modulating $T\beta 4$ activity in tissues.

THYMOSIN β4 PROMOTES WOUND REPAIR

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

This invention was made in part with funds from the National Institutes of Health, Intramural Program. The government may have certain rights in this invention.

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority from Provisional Application Serial No. 60/094,690, filed July 30, 1998, which is incorporated herein by reference in its entirety and to which application a priority claim is made under 35 U.S.C. §119(e).

TECHNICAL FIELD OF THE INVENTION

The present invention relates generally to tissue repair and more specifically to methods of wound healing using thymosin β4.

BACKGROUND OF THE INVENTION

Inadequate methods and compositions to effectively heal chronic wounds is a significant health care problem. Impaired wound healing increases the chances of mortality and morbidity. This problem is especially prominent in patients with diabetes who develop severe, life threatening wounds on body extremities. Chronic diabetic foot ulcers often lead to amputations. These wounds are often the result of poor circulation derived from the diabetic patients' insulin-compromised cells as well as impaired vascularization of the wound bed, reduced infiltration of germ fighting cells and reduced tissue epithelialization. As a result, most current therapies include attempts to revascularize the wound bed and prevent infection.

Wounds in non-compromised tissues undergo a complex and ordered series of events to repair the tissue. The series of events may include infiltration of immune cells as part of the process to remove and destroy necrotic tissue, increased vascularization by angiogenic factors and increased cell proliferation and extracellular matrix deposition. Although the basic process of tissue repair has been characterized,

been shown to increase collagen formation, DNA content, and protein levels in animal studies. (Grotendorst, GR *et al.* (1985) *J. Clin. Invest.* 76:2323-2329.; Sporn, MB *et al.* (1983) *Science* 219:1329). The effect of PDGF in wound healing has been shown to be effective in human wounds. In human wounds, PDGF-AA expression is increased within pressure ulcers undergoing healing. The increase of PDGF-AA corresponds to an increase in activated fibroblasts, extracellular matrix deposition, and active vascularization of the wound. Furthermore, such an increase in PDGF-AA is not seen in chronic non-healing wounds. A number of other growth factors having the ability to induce angiogenesis and wound healing include, Vascular Endothelial Growth Factor (VEGF), Keratinocyte Growth Factor (KGF) and basic Fibroblast Growth Factor (bFGF).

However, most of these growth and angiogenic factors have side effects. Accordingly, there is a need for additional factors useful in promoting wound repair.

SUMMARY OF THE INVENTION

The present invention is based on the discovery that thymosin β4 (Tβ4) accelerates wound healing and stimulates wound repair. Based on this finding, it is now possible to develop methods for accelerating wound healing in subjects having wounds in need of such treatment.

In a first embodiment, the invention provides a method for promoting wound repair in a subject in need of such treatment by administering to the subject or contacting the site of the wound with a wound-healing effective amount of a composition containing a wound healing polypeptide comprising the amino acid sequence LKKTET and conservative variants thereof having wound healing activity. In one aspect of the method, the wound healing polypeptide is Tβ4 or an isoform of Tβ4.

In another embodiment, the invention provides a method for promoting tissue repair in a tissue in need of such treatment by contacting the tissue with an effective amount of a composition containing a wound healing polypeptide comprising the amino acid sequence LKKTET and conservative variants thereof

DESCRIPTION OF DRAWINGS

FIG. 1 is a schematic drawing of a wound.

FIG. 2 is a bar graph which shows the effect of topical and systemic delivery of Tβ4 on the width of a punch wound as compared to control. (A) Topical delivery of 5 μg/50 μl was performed on three of the six wounds in each animal on the day of wounding and at 48 hours after wounding. (B) Intraperitoneal injections of 60 μg/300μl were done on the day of the wounding and thereafter every other day. Control animals were treated similarly with saline. Measurements are expressed as the mean percent decrease ± SEM.

FIG. 3 is a bar graph which shows the effect of topical and systemic delivery of Tβ4 on the gap of a punch wound as compared to control. (A) Topical delivery of 5 μg/50 μl was performed on the day of wounding and at 48 hours after wounding. (B) Intraperitoneal injections of 60 μg/300μl were done on the day of the wounding and thereafter every other day. Measurements are expressed as the mean percent decrease ± SEM.

FIG. 4 is a histological section, stained with H&E, demonstrating the appearance of control and thymosin β4 treated wounds at low magnification and higher magnification. Wounds are from day 7 as described in the legend to figure 2. Arrows indicate the edges of the original wound. (A) Control wound treated with saline. Migration of the epithelium is visible at the wound edges and debris are visible over the unhealed wound. (B) Increased re-epithelialization of the wound occurred when Tβ4 was injected intraperitoneally (60 μg/300μl on alternate days). (C) Topical treatment (5μg/50μl of Tβ4) resulted in complete reepithelialization of the wound epidermis. Boxed areas are the location of the higher magnification fields (D-F). (D-F) Dermis near dermal and epidermal junction. (D) Control showing few cells near the dermis and little neovascularization. (E) and (F) Dermis showing granulation tissue infiltrated with fibroblasts and extensive neovascularization (arrowheads). (E) Intraperitoneal treatment and (F) topical application both resulted in significant new capillaries. (Scale bar = 1 mm).

protein including a role in endothelial cell differentiation and migration, T cell differentiation, actin sequestration and vascularization. One biological activity of thymosin β4 (Tβ4), as shown herein, effects tissue repair and wound healing. Another activity of Tβ4 is anti-inflammatory activity.

The present invention resulted from investigation of the effects of Tβ4 on wound healing. *In vivo* results have demonstrated that topical and systemic delivery of Tβ4 promotes wound healing. Additional experiments demonstrated that Tβ4-treated wounds have increased extracellular matrix deposition in the wound bed.

The present invention identifies Tβ4 as an active factor in promoting wound closure and tissue repair *in vivo* as well as increasing epithelial cell migration. *In vivo* administration of Tβ4 indicates that cell migration, angiogenesis and extracellular matrix deposition are stimulated at or above the levels observed for migration, angiogenesis and matrix deposition in control animals. Tβ4 promotes wound closure when administered systemically (*e.g.*, intra-peritoneally) and topically in wounded animal models. Increased levels of collagen were also observed in treated wounds showing that Tβ4 treatment can also accelerate wound contraction and stimulate the healing process.

The methods of the invention result from the identification of the effect of Tβ4 on wound healing. *In vivo*, Tβ4 stimulates wound healing in a full thickness punch wound (see Example 1) and in repair of eye-related wounds (Example 4). When given either topically or systemically (*e.g.*, intra-peritoneally) Tβ4 accelerated closure and healing of wounds (see Example 1, 4, and 5).

Promoting Tissue Regeneration

In one embodiment, the invention provides a method for accelerating wound healing in a subject by contacting a wound with a wound-healing effective amount of a composition which contains Tβ4 or a Tβ4 isoform. The contacting may be topically or systemically. Examples of topical administration include, for example, contacting the wound with a lotion, salve, gel, cream, paste, spray, suspension, dispersion, hydrogel, ointment, or oil comprising Tβ4. Systemic administration

Modulation of Wound Healing

Wound healing, tissue regeneration and tissue repair result from a complex process that includes the proliferation and migration of inflammatory cells, endothelial cells, stromal cells and parenchymal cell, the deposition of extracellular matrix materials and the growth of new blood vessels, particularly capillaries. This complex process plays a crucial role in such beneficial functions as embryogenesis, the female reproductive cycle, as well as such abnormal functions as arthritis, chronic ulcerations and neuro-degenerative diseases.

In another embodiment, the invention provides a method for modulating wound healing in a subject or a tissue including contacting the subject or tissue with an effective wound-healing amount of a composition containing Tβ4 or a Tβ4 isoform. It is envisioned that Tβ4 or a Tβ4 isoform can be administered topically or systemically to prevent or treat a damaged tissue including, for example, tissues damaged due to ischemia, including ischemic brain, bone and heart disease, damage to corneal or retinal tissue of the eye, and damage to epithelial tissue, including skin.

In addition, the method of the invention is useful in promoting wound healing in tissues by promoting angiogenesis in tissue deprived of adequate blood flow. For example, a composition containing Tβ4 can promote the healing of chronic ulcers by increasing blood supply to the tissue site as well as increasing keratinocyte migration to close a wound.

Tβ4 isoforms have been identified and have about 70%, or about 75%, or about 80% or more homology to the amino acid sequence of Tβ4 set forth in Fig. 10. Such isoforms include, for example, $T\beta4^{ala}$, Tβ9, Tβ10, Tβ11, Tβ12, Tβ13, Tβ14 and Tβ15 (Fig. 11; see also, Mihelic *et al.*, (1994) *Amino Acids*, 6:1-13, which describes the amino acid sequence of other Tβ4 isoforms, and is incorporated herein by reference). Similar to Tβ4, the Tβ10 and Tβ15 isoforms have been shown to sequester actin. Tβ4, Tβ10 and Tβ15, as well as these other isoforms share an amino acid sequence, LKKTET, that appears to be involved in mediating actin sequestration or binding. Although not wishing to be bound to any particular theory, the wound healing activity of Tβ4 and Tβ4 isoforms may be due, in part, to the ability to

As used herein, the term "conservative variant" or grammatical variations thereof denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the replacement of a hydrophobic residue such as isoleucine, valine, leucine or methionine for another, the replacement of a polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like.

Tβ4 has been localized to a number of tissue and cell types and thus, agents which stimulate the production of Tβ4 can be added to a composition to effect Tβ4 production from a tissue and/or a cell. Agents that effect wound repair can also be included in such a composition to augment the wound healing process. Such agents include members of the family of growth factors, such as insulin-like growth factor (IGF-1), platelet derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor beta (TGF-β), basic fibroblast growth factor (bFGF), thymosin α1 (Tα1) and vascular endothelial growth factor (VEGF). More preferably, the agent is transforming growth factor beta (TGF-β) or other members of the TGF-β superfamily. Tβ4 compositions of the invention aid in wound healing by effectuating growth of the connective tissue through extracellular matrix deposition, cellular migration and vascularization of the wound bed.

Additionally, agents that assist or stimulate the wound healing process may be added to a composition along with Tβ4 or a Tβ4 isoform to further modulate the wound healing process. Such agents include angiogenic agents, growth factors, agents that direct differentiation of cells, agents that promote migration of cells and agents that stimulate the provision of extracellular matrix materials in the wound bed. For example, and not by way of limitation, Tβ4 or a Tβ4 isoform alone or in combination can be added in combination with any one or more of the following agents: VEGF, KGF, FGF, PDGF, TGFβ, IGF-1, IGF-2, IL-1, prothymosin α and thymosin α1 in a wound-healing effective amount.

In another aspect, the invention is useful for repair of tissue resulting from injuries due to surgical procedures, irradiation, laceration, toxic chemicals, viral infections, bacterial infections or burns. Additionally, the invention is useful for

later implantation inside the body, or augment the design and growth of a tissue inside the body to repair or replace diseased or damaged tissue. For example, Tβ4 may be useful in promoting the growth of skin graft replacements which are used as a therapy in the treatment of burns and ulcers.

In another aspect of tissue engineering, Tβ4 can be included in external or internal devices containing human tissue designed to replace the function of a diseased internal tissue. This approach involves isolating cells from the body, placing them on or within a three-dimensional matrices and implanting the new system inside the body or using the system outside the body. The methods and compositions of the invention can be used and included in such matrices to promote the growth of tissues contained in the matrices. For example, Tβ4 can be included in a tissue engineered construct to promote the growth of the cells contained in the construct. It is envisioned that the method of the invention can be used to augment tissue repair, regeneration and engineering in endothelial cell-related products which may contain cartilage, cartilage-bone composites, bone, central nervous system tissues, muscle, liver, pancreatic islet (insulin-producing) cells, urogenital tissues, breast and tissues for gene therapy applications.

The present invention further provides methods and compositions for modulating female reproductive tract function. Growth factors have been shown to play a role in cyclic mitosis and differentiation of endometrial cellular components, recruitment of macrophages in decidualizing the endometrium, endometrial-trophoblast interactions, early pregnancy maintenance, and endometrial functional regeneration. The term "modulate" as used herein, denotes a modification of an existing condition or biologic state. Modulation of a condition as defined herein, encompasses both an increase or a decrease in the determinants affecting the existing condition. For example, administration of Tβ4 could be used to augment uterine functions in a condition where the promotion of endothelial cell growth is desired. For example, the uterus may be treated with Tβ4 to promote the growth and development of placental membranes or endometrial growth or the repair of these tissue following tissue injury. Furthermore, treatment with Tβ4 may be used to

polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphtalenes, and the like. In particular, biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxethylene-polyoxypropylene copolymers are examples of excipients for controlling the release of a compound of the invention *in vivo*. Other suitable parenteral delivery systems include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation administration may contain excipients such as lactose, if desired. Inhalation formulations may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or they may be oily solutions for administration in the form of nasal drops. If desired, the compounds can be formulated as a gel to be applied intranasally. Formulations for parenteral administration may also include glycocholate for buccal administration.

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Particularly useful are diacylphosphatidylglycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidyl-choline.

The targeting of liposomes has been classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal capillaries. Active

The invention also includes a pharmaceutical composition comprising a therapeutically effective amount of Tβ4 or a Tβ4 isoform in a pharmaceutically acceptable carrier. Such carriers include those listed above with reference to parenteral administration.

The actual dosage or reagent, formulation or composition that modulates a tissue repair process, fibrotic disorder, a sclerotic disorder, a cell proliferative disorder, or wound healing depends on many factors, including the size and health of a subject. However, one of ordinary skill in the art can use the following teachings describing the methods and techniques for determining clinical dosages (Spilker B., *Guide to Clinical Studies and Developing Protocols*, Raven Press Books, Ltd., New York, 1984, pp. 7-13, 54-60; Spilker B., *Guide to Clinical Trials*, Raven Press, Ltd., New York, 1991, pp. 93-101; Craig C., and R. Stitzel, eds. , *Modern Pharmacology*, 2d ed., Little, Brown and Co., Boston, 1986, pp. 127-33; T. Speight, ed., *Avery's Drug Treatment: Principles and Practice of Clinical Pharmacology and Therapeutics*, 3d ed., Williams and Wilkins, Baltimore, 1987, pp. 50-56; R. Tallarida, R. Raffa and P. McGonigle, *Principles in General Pharmacology*, Springer-Verlag, New York, 1988, pp. 18-20) or to determine the appropriate dosage to use.

Antibodies that Bind to Tβ4

Antibodies to Tβ4 peptide or fragments could be valuable as diagnostic tools to aid in the detection of diseases in which Tβ4 is a pathological factor. Further, use of antibodies which bind to Tβ4 and inhibit or prevent the actions of Tβ4 are included in the present invention. Therapeutically, antibodies or fragments of the antibody molecule could also be used to neutralize the biological activity of Tβ4 in diseases where Tβ4 is over expressed. Such antibodies can recognize an epitope of Tβ4 or fragments thereof suitable for antibody recognition and neutralization of Tβ4 activity. As used in this invention, the term "epitope" refers to an antigenic determinant on an antigen, such as a Tβ4 peptide, to which the paratope of an antibody, such as an Tβ4-specific antibody, binds. Antigenic determinants usually consist of chemically active surface groupings of molecules, such as amino acids or

The invention provides a method for detecting Tβ4, or variants thereof, which includes contacting an anti-Tβ4 antibody with a sample suspected of containing Tβ4, (*e.g.*, cell or protein) and detecting binding to the antibody. An antibody which binds to Tβ4 peptide is labeled with a compound which allows detection of binding to Tβ4. There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, phosphorescent compounds, and bioluminescent compounds. Those of ordinary skill in the art will know of other suitable labels for binding to the antibody, or will be able to ascertain such, using routine experimentation. For purposes of the invention, an antibody specific for Tβ4 peptide may be used to detect the level of Tβ4 in biological fluids and tissues. Any specimen containing a detectable amount of antigen can be used. The level of Tβ4 in the suspect cell can be compared with the level in a normal cell to determine whether the subject is predisposed to a Tβ4 associated increase in angiogenesis or wound healing.

Use of antibodies for the diagnostic methods of the invention includes, for example, immunoassays in which the antibodies can be utilized in liquid phase or bound to a solid phase carrier. In addition, the antibodies in these immunoassays can be detectably labeled in various ways. Examples of types of immunoassays which can utilize antibodies of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay (RIA) and the sandwich (immunometric) assay. Detection of the antigens using the antibodies of the invention can be done utilizing immunoassays which are run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples. Those of skill in the art will know, or can readily discern, other immunoassay formats without undue experimentation.

(3) An $(Fab')_2$ fragment of an antibody can be obtained by treating a whole antibody molecule with the enzyme pepsin, without subsequent reduction. A $(Fab')_2$ fragment is a dimer of two Fab' fragments, held together by two disulfide bonds.

(4) An Fv fragment is defined as a genetically engineered fragment containing the variable region of a light chain and the variable region of a heavy chain expressed as two chains.

(5) A single chain antibody ("SCA") is a genetically engineered single chain molecule containing the variable region of a light chain and the variable region of a heavy chain, linked by a suitable, flexible polypeptide linker.

Alternatively, a therapeutically or diagnostically useful anti-Tβ4 antibody may be derived from a "humanized" monoclonal antibody. Humanized monoclonal antibodies are produced by transferring mouse complementary determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain, and then substituting human residues in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are described, for example, by Orlandi *et al., Proc. Natl. Acad. Sci. USA* 86: 3833 (1989), which is hereby incorporated in its entirety by reference. Techniques for producing humanized monoclonal antibodies are described, for example, by Jones *et al.*, *Nature* 321: 522 (1986); Riechmann *et al.*, *Nature* 332: 323 (1988); Verhoeyen *et al., Science* 239: 1534 (1988); Carter *et al., Proc. Nat'l Acad. Sci. USA* 89: 4285 (1992); Sandhu, *Crit. Rev. Biotech.* 12: 437 (1992); and Singer *et al., J. Immunol.* 150: 2844 (1993), which are hereby incorporated by reference.

Antibodies of the invention also may be derived from human antibody fragments isolated from a combinatorial immunoglobulin library. See, for example, Barbas *et al.*, METHODS: A COMPANION TO METHODS IN ENZYMOLOGY, VOL. 2, page 119 (1991); Winter *et al., Ann. Rev. Immunol.* 12: 433 (1994), which are hereby incorporated by reference. Cloning and expression vectors that are useful

high level of Tβ4, the administration of an agent, such as an antagonist of Tβ4 activity, may be effective in decreasing the amount of Tβ4 activity. Alternatively, where the disease is due to an abnormally low level of Tβ4, the administration of Tβ4 or an agent that increases Tβ4 activity, such as an agonist, may be effective in increasing the amount of Tβ4 activity.

In yet another embodiment, the invention provides a method of treating a subject having a wound healing disorder characterized by recurrent or slow to heal wounds or wounds that are chronic non-healing wounds associated with altered Tβ4 or Tβ4 isoform gene expression in a subject. The method includes administering to a subject having the disorder a wound-healing effective amount of an agent which modulates Tβ4 gene expression, thereby treating the disorder. The term "modulate" refers to inhibition or suppression of Tβ4 expression when Tβ4 is over expressed, and induction of expression when Tβ4 is under expressed. The term "wound-healing effective amount" means that amount of Tβ4 agent which is effective in modulating Tβ4 gene expression resulting in reducing the symptoms of the Tβ4 associated wound healing disorder.

An agent which modulates Tβ4 or Tβ4 isoform gene expression may be a polynucleotide for example. The polynucleotide may be an antisense, a triplex agent, or a ribozyme. For example, an antisense may be directed to the structural gene region or to the promoter region of Tβ4 may be utilized.

When a wound healing disorder is associated with the expression of Tβ4, a therapeutic approach which directly interferes with the translation of Tβ4 mRNA into protein is possible. For example, an antisense nucleic acid or a ribozyme can be used to bind to the Tβ4 RNA or to cleave it. Antisense RNA or DNA molecules bind specifically with a targeted gene's RNA message, interrupting the expression of that gene's protein product. The antisense binds to the mRNA forming a double stranded molecule which cannot be translated by the cell. Antisense oligonucleotides of about 15-25 nucleotides are preferred since they are easily synthesized and have an inhibitory effect just like antisense RNA molecules. In addition, chemically reactive group, such as iron-linked ethylenediaminetetraacetic acid (EDTA-Fe) can be

recognition sequence, the greater the likelihood that the sequence will occur exclusively in the target mRNA species.

These and other uses of antisense methods to inhibit the *in vivo* translation of genes are well known in the art (*e.g.*, De Mesmaeker, *et al.*, 1995. Backbone modifications in oligonucleotides and peptide nucleic acid systems. *Curr. Opin. Struct. Biol.* 5:343-355; Gewirtz, A.M., *et al.*, 1996b. Facilitating delivery of antisense oligodeoxynucleotides: Helping antisense deliver on its promise; *Proc. Natl. Acad. Sci. U.S.A.* 93:3161-3163; Stein, C.A. A discussion of G-tetrads 1996. Exploiting the potential of antisense: beyond phosphorothioate oligodeoxynucleotides. *Chem. and Biol.* 3:319-323).

Delivery of antisense, triplex agents, ribozymes, competitive inhibitors and the like can be achieved using a recombinant expression vector such as a chimeric virus or a colloidal dispersion system. Various viral vectors which can be utilized for gene therapy as taught herein include adenovirus, herpes virus, vaccinia, or, preferably, an RNA virus such as a retrovirus. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated. By inserting a polynucleotide sequence of interest into the viral vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target specific. Retroviral vectors can be made target specific by inserting, for example, a polynucleotide encoding a sugar, a glycolipid, or a protein. Preferred targeting is accomplished by using an antibody to target the retroviral vector. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific polynucleotide sequences which can be inserted into the retroviral genome to allow target specific delivery of the retroviral vector containing the antisense polynucleotide.

of interest at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, *et al., Biotechniques*, 6:682, 1988).

Pathologically, Tβ4 may be involved in diseases in which there is an overgrowth of blood vessels, such as cancer, tumor formation and growth, diabetic retinopathy, neovascular glaucoma, rheumatoid arthritis and psoriasis.

The ingrowth of capillaries and ancillary blood vessels is essential for growth of solid tumors and is thus an unwanted physiological response which facilitates the spread of malignant tissue and metastases. Inhibition of angiogenesis and the resultant growth of capillaries and blood vessels is therefore a component of effective treatment of malignancy in use of treatment of cancer patients.

Thus, in another embodiment, the invention provides a method of inhibiting angiogenesis in a subject, including administering to the subject a composition containing an agent which regulates Tβ4 activity. The composition may include agents that regulate angiogenesis, for example agents that affect thymosin α1, PDGF, VEGF, IGF, FGF and TGFβ. For example, the inhibition of angiogenesis and endothelial cell migration can be beneficial in controlling the growth of solid tumors. Most, if not all solid tumors, like normal tissue, require a steady and sufficient blood supply for optimal growth. Tumors are known to make use of angiogenic growth factors to attract new blood vessels and ascertain supply with sufficient amounts of nutrients to sustain their growth. Many tumors are well vascularized and the inhibition of the formation of an adequate blood supply to the tumor by inhibition of tumor vascularization, as a result of inhibition of angiogenesis, is beneficial in tumor growth control. Without a strong blood supply, rapid and prolonged growth of tumor tissue cannot be sustained. Thus, agents that inhibit Tβ4 activity may be used to prevent neoplastic growth. The Tβ4 inhibiting agent may be administered orally, parenterally, topically, intravenously, or systemically. In addition, for inhibiting tumor cell proliferation and tumor growth, the agent may be administered locally

as zincs, and biological agents. Tβ4 activity can be assayed using the methodology as described in the present Examples.

The present Examples are meant to illustrate, but not limit the scope of the appended claims. Accordingly, one skilled in the art will recognize a number of equivalent materials and methods, which are intend to be covered by the present invention and disclosure.

EXAMPLE 1

In vivo wound healing is accelerated by Tβ4

Tβ4, whether administered topically or intraperitoneal, significantly accelerated wound healing as compared to untreated wounds (Fig. 2 and 3). Full thickness 8 mm punch biopsy wounds were made on the dorsal surface of rats as previously reported (Bhartiya *et al., J. Cell. Physiol.* 150:312, 1992; Sihhu *et al., J. Cell. Physiol.* 169:108, 1996) and Tβ4 was given topically at the time of wounding (5 μg in 50 μl) and again after 48 hours. Controls for the topical treatment received identical amounts of saline at the time of wounding and at 48 hours. Additional rats received intraperitoneal injections at the time of wounding (60 μg in 300 μl) and again every other day (*e.g.*, days 0, 2, 4, and 6). Controls for these animals received identical amounts of saline intra-peritoneally on the same injection schedule. On days 4 and 7 post-wounding, measurements were made on the wound size. At days 8 and 9 post-wounding, tissue was collected and fixed in 10% buffered formalin. The samples were sectioned and stained with H&E and Masson's Trichrome (American Histolabs, Gaithersburg, MD).

Histological sections were used to measure the re-epithelialization and the contraction of the wound using an ocular micrometer. Epidermal migration was determined by measuring the lengths of the tongues of epithelium migrating form either side of the wound over the wound bed from the zone of proliferation at the margin of the uninjured and wounded skin. Epidermal thickness was also measured beginning at the junction of the uninjured and proliferating epidermis. The thickness was measured vertically from the basement membrane to the most superficial layer of

wounds treated for 7 days and the rate of gap closure was slightly accelerated over that observed at day 4. A 62% decrease in gap size was observed in the Tβ4-treated wounds. Quantitation of epidermal migration showed a statistically significant 1.5 fold increase in migration of epidermal tongues over the wound bed after topical treatment (Table 1). Quantitation of epithelial migration in intraperitoneally treated wounds showed a statistically significant increase in migration of epidermal tongues as compared to controls (Table 1). There was no difference in the thickness of the migrating epidermis between either of the Tβ4 treatments and the control (Table 1). Histological sections of the wounds clearly show increased re-epithelialization in the treated wounds as compared to controls in 7 day wounds (FIG 4).

Table 1: Morphometric Measurements of Control and Thymosin β4 Treated Samples

Parameter	Control	I.P.	Topical
Epidermal Migration (μm)	2403.3±9.7	3168.3±38.4*	3668.7±56.6*
Epidermal Thickness (μm)	128.2±19.3	135.0±11.7	142.3±19.8
Vessels/10 HPF	1364.0±15.0	2415.0±24.3*	2186.0±11.8*

HPF: high power field. *$P \leq 0.00001$ by Welch's t-test, significantly different than control.

FIG. 4 shows a comparison of typical control (D) and Tβ4-treated (E and F) sections of 7 day wounds. Treatment with Tβ4 resulted in considerable capillary ingrowth (FIG 4E and F, arrows). Vessel counts showed a significant (about 2 fold) increase in the number of vessels in Tβ4 treated wounds (Table 1). No increases in the number of macrophages in the wounds were observed. There was no apparent increase in the accumulation/biosynthesis of collagen in treated -Tβ4 wounds (Fig. 5B and C vs A) supporting a decreased wound width and supporting a role for Tβ4 in wound contraction. Both the topical and systemically treated wound appeared similar although the wound contraction proceeded slightly more quickly with the topical treatment.

then fixed and stained using Diff-Quik (Baxter Healthcare Corporation, McGraw Park, IL). The cells that migrated through the filter were quantitated by counting the center of each well at 10x using an Olympus CK2 microscope. Each condition was assayed in triplicate wells and each experiment was repeated four times with different preparations of cells.

The results demonstrated that keratinocyte migrated in response to Tβ4 after 4-5 hours of exposure. Migration was enhanced 2-3 fold ($P \leq 0.003$) over migration in the presence of media alone (FIG. 6) and at the maximal responding dose exceeded the positive control. The effect of Tβ4 on migration, while showing slightly different dose curves depending on the cell preparation and source, clearly showed a biphasic pattern with 1000 ng/ml and 0.01 ng/ml showing the most migration and the middle doses showing less stimulation (but still greater than control media) in all 4 assays.

EXAMPLE 3

Migration Assays of Corneal Epithelial Cells

Corneal Epithelial Cell migration assays were carried out in Boyden chamber using 12 μm pore polyester membranes (Poretics, Livermore, CA) coated with a 0.1 mg/ml solution of collagen IV in dH20 (Trevigen, Gaithersburg, MD). Filters were then dried at least 1 h. Cells were cultured and resuspended in Eagle's Minimal Essential Medium with 0.05 mM Ca^{2+}. The bottom chamber was loaded with EMEM containing 0.01, 0.1, 10, 100, and 1000 ng/ml of synthetic Tβ4. Conditioned medium from primary dermal fibroblasts and/or keratinocyte growth factor was added to several wells as a positive control. Cells were added to the upper chamber at a concentration of 50,000 cells per well. Chambers were incubated at 35 C/7% CO_2 for 4-5 hours and the filters were then fixed and stained using Diff-Quik (Baxter Healthcare Corporation, McGraw Park, IL). The cells that migrated through the filter were quantitated by counting the center of each well at 10x using an Olympus CK2 microscope. Each condition was assayed in triplicate wells and each experiment was repeated four times with different preparations of cells.

The gap in topically treated animals decreased by 39% compared to saline treatment. The wound width decreased by 23%. Intraperitoneal injection resulted in a 26% decrease in gap size and a 10% decease in wound width. Taken together, these demonstrate that Tβ4 is useful to treat chronic, as well as, acute wounds.

A number of embodiments of the present invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

9. The method of claim 2, wherein the isoform of thymosin β4 is at least 70% homologous to thymosin β4 peptide set forth as SEQ ID NO:1 in Figure 10.

10. The method of claim 9, wherein the isoform of thymosin β4 is selected from the group consisting of: $T\beta4^{ala}$, Tβ9, Tβ10, Tβ11, Tβ12, Tβ13, Tβ14 and Tβ15.

11. The method of claim 1, further comprising contacting the site of the wound with an agent which promotes wound healing.

12. The method of claim 11, wherein the agent is selected from the group consisting of IGF, IGF-1, IGF-2, IL-1, PDGF, FGF, KGF, VEGF, prothymosin α, thymosin α1 or combinations thereof.

13. A method for promoting wound healing in a subject in need of such treatment comprising administering to the subject a wound-healing effective amount of a composition containing thymosin β4 or an isoform of thymosin β4.

14. The method of claim 13, wherein the composition further contains an agent that stimulates the production of thymosin β4 peptide.

15. The method of claim 14, wherein the agent is transforming growth factor beta (TGF-b).

16. The method of claim 13, wherein the thymosin β4 is delivered systemically.

17. The method of claim 13, wherein the thymosin β4 is delivered topically.

18. The method of claim 17, wherein the thymosin β4 is contained in a topical formulation selected from the group consisting of a gel, cream, paste, lotion, spray, suspension, dispersion, salve, hydrogel and ointment.

30. The method of claim 29, wherein the agent is transforming growth factor beta (TGF-b).

31. The method of claim 29, wherein the agent is a mineral.

32. The method of claim 29, wherein the mineral is zinc.

33. The method of claim 23, wherein the wound healing polypeptide is delivered topically.

34. The method of claim 23, wherein the wound healing polypeptide is contained in a topical formulation selected from the group consisting of a gel, cream, paste, lotion, spray, suspension, dispersion, salve, hydrogel and ointment.

35. The method of claim 23, wherein the wound healing polypeptide is delivered systemically.

36. The method of claim 23, further comprising contacting the site of the tissue with an agent which promotes wound healing.

37. The method of claim 36, wherein the agent is selected from the group consisting of IGF, IGF-1, IGF-2, PDGF, FGF, KGF, VEGF, prothymosin α, thymosin $\alpha 1$ or combinations thereof.

38. The method of claim 23, wherein the tissue is selected from the group consisting of epidermal, eye, uro-genital, gastro-intestinal, cardiovascular, muscle, connective, and neural.

39. The method of claim 23, wherein the tissue is skin tissue.

48 The method of claim 47, wherein the thymosin β4 regulating agent is an antagonist of thymosin β4 peptide.

49. The method of claim 48, wherein the antagonist is an antibody which specifically binds to thymosin β4 peptide.

50. A method for identifying a compound which modulates wound healing, angiogenesis or cell migration activity, comprising contacting thymosin β4 or an isoform of thymosin β4 with a compound suspected of having thymosin β4 modulating activity and detecting an effect on thymosin β4 or thymosin β4 isoform activity.

51 The method of claim 50, wherein the compound is an agonist of thymosin β4 activity.

52. The method of claim 50, wherein the compound is an antagonist of thymosin β4 activity.

53 A method of promoting epithelial cell migration, comprising contacting an epithelial cell with a composition comprising thymosin β4 or an isoform of thymosin β4.

54. The method of claim 53, wherein the epithelial cell is a skin cell.

55. The method of claim 54, wherein the skin cell is a keratinocyte.

56. The method of claim 53, wherein the epithelial cell is a corneal epithelial cell.

57. The method of claim 53, wherein the contacting is *in vivo*.

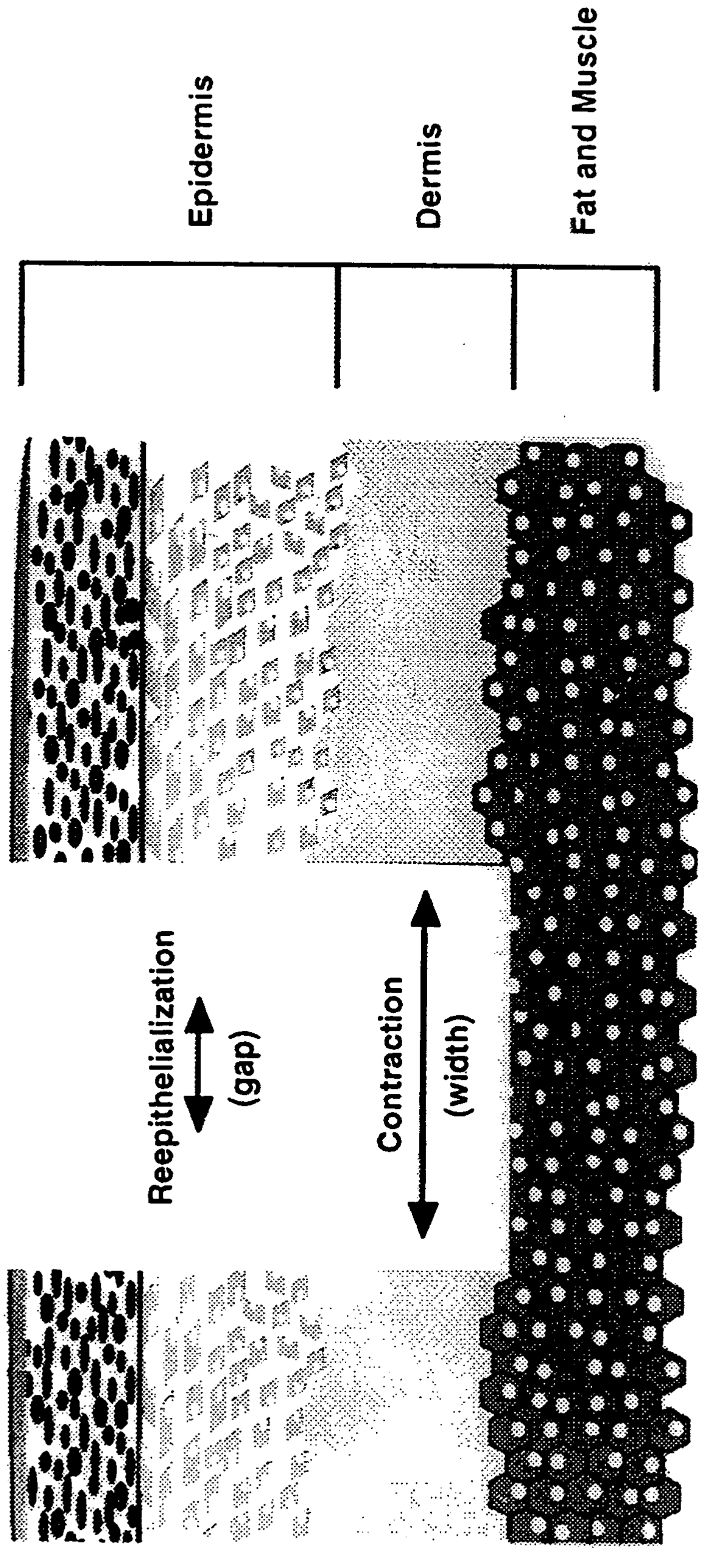

Epidermis
Dermis
Fat and Muscle
Reepithelialization
(gap)
Contraction
(width)

FIG. 1

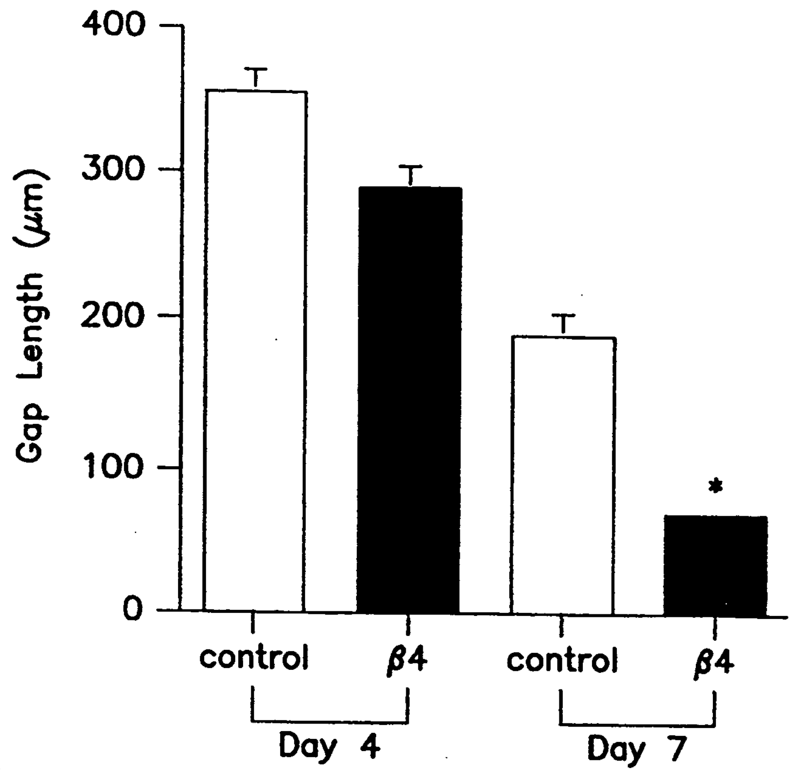

400
300
200
100
0
Gap Length (μm)
control
β4
control
β4
Day 4
Day 7
*

FIG. 3A

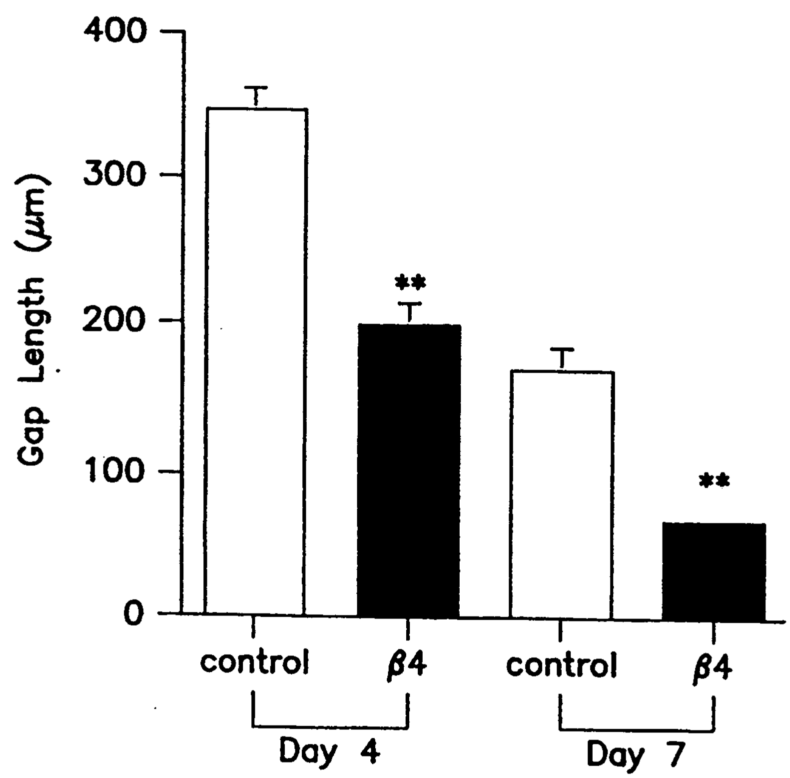

400
300
200
100
0
Gap Length (μm)
**
**
control
β4
control
β4
Day 4
Day 7

FIG. 3B

FIG. 5a

FIG. 5b

FIG. 5c

FIG. 5d

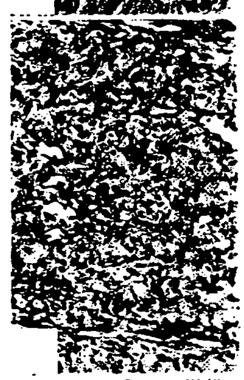
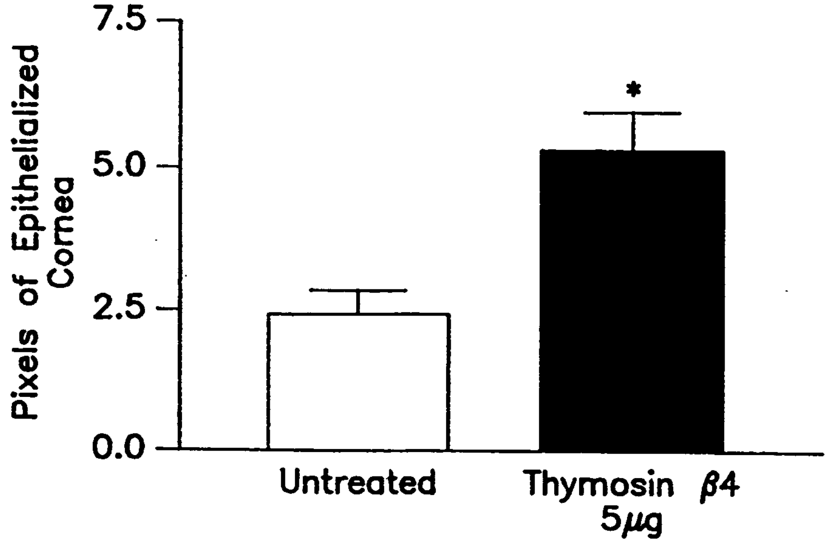


FIG. 5e

FIG. 5f

Thymosin β4 Stimulat s
Corneal R -epithelialization in
the Rat Cornea at 24 Hours



*p=0.003

n=6

FIG. 8

Thymosin β4 Stimulates
Re-epithelialization in the Rat
Cornea at 24 Hours:
Dose Response Experiment

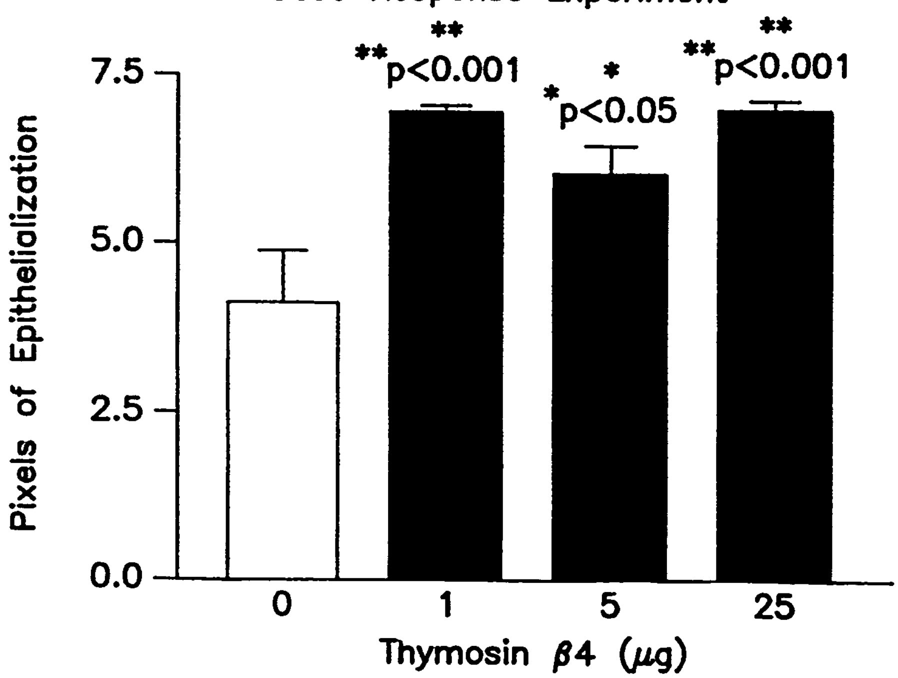


FIG. 9

Structural Formula of Thymosin Beta 4

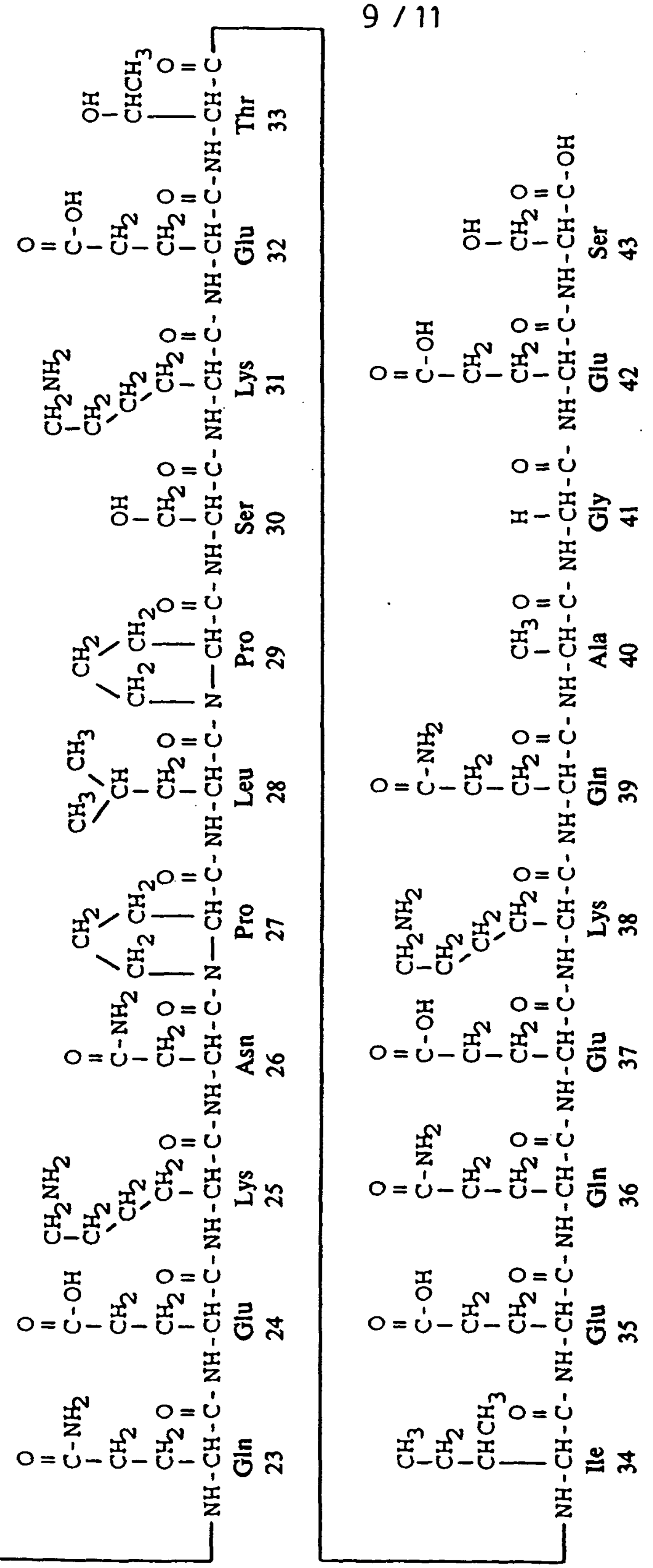


FIG. 10b

Phylogenetic Distribution of Thymosin β_4-Like Peptides

Species	First peptide	Second peptide	Third peptide
Human	β_4	β_{10}	β_{15}
Rat, mouse, cat	β_4	β_{10}	β_{15} (rat tumor)
Calf	β_4	β_9	
Pig, sheep	β_4	β_9^{Met}	
Horse, chicken, gecko	β_4		
Xenopus laevis	β_4^{Xen}		
Rainbow trout	β_{11}	β_{12}	
Perch	β_{12}^{perch}		
Whale	β_{13}		
Sea urchin	β_{14}	$\beta^{sea\ urchin}$	
Scallop	$\beta^{scallop}$		

FIG. 11b

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/17282

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SUN H -Q ET AL: "BETA-THYMOSINS ARE NOT SIMPLE ACTIN MONOMER BUFFERING PROTEINS. INSIGHTS FROM OVEREXPRESSION STUDIES" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 16, April 1996 (1996-04), pages 9223-9230, XP002041936 ISSN: 0021-9258 page 9223, column 1, line 15 - line 18 page 9229; figure 3	1,2, 8-10,13, 19-21, 23,24, 26,53, 60,62,63
X	page 9224, section "Quantitative Immunoblotting"	45,46
Y	page 9227, column 1, line 5 -page 9228, column 1, line 3	11,22,36

Y	NIMNI M E: "Polypeptide growth factors: targeted delivery systems" BIOMATERIALS, vol. 18, no. 18, 1997, pages 1201-1225, XP004086390 ISSN: 0142-9612 page 1210, column 1, line 15 - line 53 page 1203 -page 1211	3,4,11, 12,14, 15,22, 29,30, 36,37
A	page 1205, section "Keloid and hypertrophic scars"	41-44
A	pages 1215-1216, sections "Delivery of growth factors in wound healing" and "Some novel and potentially useful approaches for local delivery of growth factors"	38-40, 54-61,64

Y	WO 96 16983 A (JOLLA CANCER RES FOUND) 6 June 1996 (1996-06-06) page 1 -page 2; claims 1,4	11,12, 22,36,37
A	claims 5,6,8,11,16-19	6,7,17, 18,33, 34,58, 61,64

Y	FRANK, S ET AL: "Regulation of vascular endothelial growth factor expression in cultured keratinocytes and implications for normal and impaired wound healing" J. BIOL. CHEM., vol. 270, no. 21, May 1995 (1995-05), pages 12607-12613, XP002125696 page 12607, column 2, line 13 - line 26 page 12609, column 2, line 7 - line 12	11,12, 22,36,37

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INTERNATIONAL SEARCH REPORT	International application No. PCT/US 99/ 17282

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 99/17282

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